



Development of somaclones in sugarcane through callus culture and subsequent molecular diversity analysis using RAPD markers

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The experiment was conducted at the Biotechnology Laboratory, Bangladesh Sugarcrop Research Institute (BSRI), Ishurdi, Pabna, Bangladesh. Sugarcane somaclones were developed using callus culture supplemented with 2,4-D. Sugarcane somaclones and their sources varieties were analyzed by RAPD molecular markers to check the variation at molecular level based. Information of genetic similarities and diversity among somaclones and their sources varieties is necessary for future breeding programs and derivation of plant lines. Genetic variability and relationships among four varieties viz. Isd 37, Isd 38, Isd 39 and Isd 40 and their 12 somaclones CC-37-1, CC-37-4, CC-37-5, CC-38-1, CC-38-2, CC-38-5, CC-39-3, CC-39-5, CC-39-6, CC-40-2, CC-40-3 and CC-40-6 were established by using six RAPD primers. The highest genetic dissimilarity was detected between Isd 37 variety and somaclone CC-37-1 (91%), while the lowest dissimilarity was found between Isd 40 variety and somaclone CC-40-6 (51%). All genotypes (somaclones and their sources varieties) showed an average genetic similarity of 0.26%. Six RAPD primers used in this experiment were able to discriminate all genotypes of sugarcane analyzed. Highest amount of DNA (6741.5 ng/μl) was recovered from the variety Isd 37 and the lowest amount (2012.5 ng/μl) was obtained from the somaclone CC-39-5 from Isd 39 variety. The highest number of bands (26) was produced from Isd 39 variety followed by somaclone CC-40-6 (23) of Isd 40 variety. Therefore, it may be concluded that callus culture supplemented with 2,4-D can be used to create somaclonal variation along with molecular diversity which can be determined by using RAPD markers.

Key words: RAPD, sugarcane, somaclones and variation

INTRODUCTION

Sugarcane (*Sccharum officinarum*) is a tropical, tall growing, monocotyledonous perennial grass, belonging to the genus *Saccharum*. Sugarcane is globally a major source of raw material for the production of commercial sugar accounting for almost two-thirds of world sugar production. It is multipurpose crop providing not only sugar, but also a series of value added products and by-products such as energy, chemicals, and single cell protein, ethanol, bio-gas, fertilizer, fibre board and paper, polishers, cosmetics and candles. Although many countries are producers, only six of them account for 65% of the world's entire sugarcane production. Among these Brazil is the largest one

(Viera, 2002). It is very urgent to increase cane productivity without further area expansion to meet the future need of sugar and gur. The chemical composition of a matured, sound and normal sugarcane stalk of the species *Saccharum officinarum* are water 74.96%, sugar 13.40%, fibre 10.04%, ash 0.64%, N₂ bodies 0.58%, fat and wax 0.38%. Sugarcane is propagated vegetatively for commercial planting by stem cuttings. Tissue culture offers an opportunity to disease free planting material. Somaclones may show variation for different parameters like yield, sugar recovery, disease resistance, drought and salt tolerance, maturity etc. It is not controversial that tissue culture tools are playing their part in sugarcane improvement and at the same time for a plant breeder assessment of genetic diversity. The cultivated sugarcane (*Saccharum spp.* hybrids) is a genetically complex polyploid grass because of its multi-species origin. Due to high level of ploidy and complex genome, the progress in deciphering sugarcane genetics has been slow. Molecular markers have become important tools in studies of genetic diversity (Bered *et al.*, 2005), due to the high resolution and reliability in the identification of cultivars. RAPD primers are able to distinguish taxa below the species level (Choo *et al.*, 2009) because RAPD analysis reflects both coding and non-coding regions of the

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genome (Vanijajiva *et al.*, 2005). Genetic markers have contributed much to understanding plant genetic diversity. Molecular markers are extensively being used to measure the variability present at genetic level, within and among the genotypes. The most commonly applied molecular markers used to study polymorphism are Random Amplified Polymorphic DNA (RAPD). The objectives of this study were to develop somaclones as well as to study the genetic variability in the somaclones compared to their sources varieties using RAPD markers.



Figure 1 Somaclone in the field

RESULTS AND DISCUSSION

Somaclones were developed in the field from four sugarcane varieties (Figure 1). The purity and quantity of the DNA extracted from the sugarcane were evaluated by using 260/280 nm Ultra Violet (UV) absorption ratios; in most cases the absorption ratio of 260/280 nm was between 1.8 and 2.18 (Table 1). The recovered amount of DNA was 2012.5–6741.5 ng/μl. The highest amount of DNA (6741.5 ng/μl) was recovered from the variety Isd 37 and the lowest amount (2012.5 ng/μl) was obtained from the somaclone CC-39-5 from Isd 39 variety. On the other hand, in case of Isd 37 variety and their somaclones, the lowest amount (2748.2 ng/μl) of DNA was recorded from CC-37-5 followed by CC-37-1 (3152.8 ng/μl), CC-37-4 (5763.8 ng/μl) and Isd 37 (6741.5 ng/μl) respectively. In case of Isd 38 variety and their somaclones, the highest amount (4891.3 ng/μl) was found from CC-38-5 followed by CC-38-1 (2256.9 ng/μl), Isd 38 (2128.2 ng/μl) and CC-38-2 (2031.4 ng/μl) respectively. Furthermore, considering Isd 39 variety and their somaclones the highest amount (3505.5 ng/μl) was recovered from CC-39-6 followed by CC-39-3 (3407.9 ng/μl), Isd 39 (3395.6 ng/μl) and CC-39-5 (2012.5 ng/μl) respectively. Besides, in case of Isd 40 variety and their somaclones, the highest amount (3842.8 ng/μl) was obtained from Isd 40 followed by CC-40-2 (3157.7 ng/μl), CC-40-6 (2868.6 ng/μl) and CC-40-3 (2852.9 ng/μl) respectively. It revealed that somaclones and their parents have different in case of amount DNA extraction and ratio at A260/A280.

The RAPD maker provided a quick and efficient for DNA sequence-based polymorphisms at a very large number of loci. The major advantage is that no prior DNA sequence information is required. DNA fingerprinting of total 16 genotypes including 4

varieties and their 12 somaclones were performed using the 6 RAPD markers. These are OPA-01, OPA-02, OPA-04, OPB-05, OPB-6 and OPA-07. The size of amplification products were estimated by comparing the migration of each amplified fragment with that of a known size fragments of molecular weight marker (100 bp DNA ladder). The sizes of the amplified bands in the 16 sugarcane genotypes ranged from 145 bp to 1000 bp. Among the six primers, two primers OPA-04 and OPB-06 produced comparatively maximum number of high intensity bands with minimal smearing and they exposed band sizes ranged from 145 bp to 1000 bp. DNA amplification (bands) out of range from 100 bp to 1000 bp were excluded from calculation. The DNA amplification of OPA-01 marker ranged from 357 bp to 711 bp, Primer OPA-02 ranged from 278 bp to 988 bp, primer OPB-05 ranged from 365 bp to 955 bp and primer OPA-07 ranged from 258 bp to 1000 bp. All distinct bands or fragments using Random Amplified Polymorphic DNA (RAPD) markers based on Agarose Gel Electrophoresis (AGE) were thereby given identification numbers according to their position on the gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. Representative electrophoregrams according to primers OPB-06 and OPA-07 were shown in Figures 1 and 2 respectively. The total number of bands (237) varied from 15 to 63 (Table 2). The highest number of bands (63) was amplified by the primer OPA-04 followed by OPB 06 (58) while the primer OPA- 01 amplified the lowest number of bands (15). The highest number of bands (3.94) per genotype was amplified from the primer OPA-04 followed by OPB-06 (3.63) and OPA-07 (2.25) respectively. The highest number of bands (26) was produced from Isd 39 variety followed by somaclone CC-40-6 (23) of Isd 40 variety (Table 1). On the other hand, the lowest number of bands (6) was obtained from the somaclone CC-40-3 of Isd 40 variety.

Parent (Variety)–Somaclone similarity indices based on RAPD markers

The four varieties and their somaclone pair wise similarity indices and dissimilarity were calculated (Table 3). Genetic similarity values defined as the fraction of shared bands between the RAPD profiles of any two individuals on the same gel were calculated manually from RAPD markers of the same molecular weight on the data matrix according to the following formula: Similarity index (SI) = $2N_{xy}/N_x + N_y$ Where, N_{xy} is the number of RAPD bands shared by individuals x and y respectively, N_x and N_y are the number of bands in individuals x and y respectively (Lynch 1990; Chapco *et al.*, 1992; Wilde *et al.*, 1992).

The SI value ranged from 0 to 1. When SI = 1.0, the two DNA profiles are identical and when SI is 0.0, there are no common bands between the two files. Within population similarity (Si) was calculated as the average of SI across all possible comparisons between individuals within a population. Between population similarity (Sij) was calculated as the average similarity between each paired individuals of population i and j (Lynch 1990). Dissimilarity index expressed in percentage is the complement of genetic similarity (i.e. Genetic Dissimilarity (DS) = 1– Genetic Similarity). The highest similarity index (0.49) along with lowest dissimilarity percentage (51%) was found in Isd 40 vs CC-40-6 followed by Isd 40 vs CC-40-2 (SI 0.43, DS 57%) while the lowest similarity index (0.09) and the highest

Table 1 Quality and Quantity assessment of DNA isolated from leaves of somaclones and their parents

Variety/ Somaclone	Concentration of DNA (ng/μl)	Absorbance Readings at 260 nm	Absorbance Readings at 280 nm	A260/A280 Ratio	A260/A230 Ratio
Isd 37	6741.5	134.83	64.33	2.10	2.10
CC-37-1	3152.8	63.65	30.68	2.05	2.20
CC-37-4	5763.8	115.27	56.01	2.06	2.13
CC-37-5	2748.2	54.96	26.50	2.07	2.04
Isd 38	2128.2	42.56	20.50	2.08	2.25
CC-38-1	2256.9	45.13	22.81	1.98	1.94
CC-38-2	2031.4	40.62	19.95	2.04	2.04
CC-38-5	4891.3	97.82	46.38	2.18	2.18
Isd 39	3395.6	67.91	32.89	2.06	2.11
CC-39-3	3407.9	68.15	34.12	2.00	1.86
CC-39-5	2012.5	40.24	20.10	2.00	1.93
CC-39-6	3505.5	70.10	34.21	2.05	2.33
Isd 40	3842.8	76.85	36.41	2.11	2.22
CC-40-2	3157.7	63.15	29.99	2.11	2.38
CC-40-3	2852.9	57.05	28.01	2.04	2.02
CC-40-6	2868.6	57.37	28.89	1.99	2.05

Table 2 Number of amplified fragments scored against 16 sugarcane genotypes using 6 RAPD primers

Primer codes	Isd 37	CC-37-1	CC-37-2	CC-37-3	Isd 38	CC-38-1	CC-38-2	CC-38-5	Isd 39	CC-39-3	CC-39-5	CC-39-6	Isd 40	CC-40-2	CC-40-3	CC-40-6	Total
OPA-01	2	0	0	1	0	0	1	1	1	0	1	0	3	3	0	2	15
OPA-02	1	1	1	1	0	0	1	0	4	2	3	3	4	2	3	4	30
OPA-04	0	8	6	1	3	5	4	4	8	0	3	5	3	6	0	7	63
OPB-05	3	3	2	3	3	1	3	1	2	0	2	2	3	2	2	3	35
OPB-06	1	0	0	6	5	9	0	5	9	5	6	2	0	5	0	5	58
OPA-07	0	3	7	7	0	1	1	3	2	1	1	1	3	3	1	2	36
Total	7	15	16	19	11	16	10	14	26	8	16	13	16	21	6	23	237
Average of six primers									39.5								

Table 3 Pair wise parent (variety) and their somaclones similarity index and dissimilarity (%) based on RAPD marker

Sl. No.	Genotype combination	Similarity index	Dissimilarity (%)
1.	Isd 37 vs CC-37-1	0.09	91
2.	Isd 37 vs CC-37-4	0.11	89
3.	Isd 37 vs CC-37-5	0.12	88
4.	Isd 38 vs CC-38-1	0.27	73
5.	Isd 38 vs CC-38-2	0.16	84
6.	Isd 38 vs CC-38-5	0.22	78
7.	Isd 39 vs CC-39-3	0.27	73
8.	Isd 39 vs CC-39-5	0.27	73
9.	Isd 39 vs CC-39-6	0.38	62
10.	Isd 40 vs CC-40-2	0.43	57
11.	Isd 40 vs CC-40-3	0.18	72
12.	Isd 40 vs CC-40-6	0.49	51
Average		0.26	74

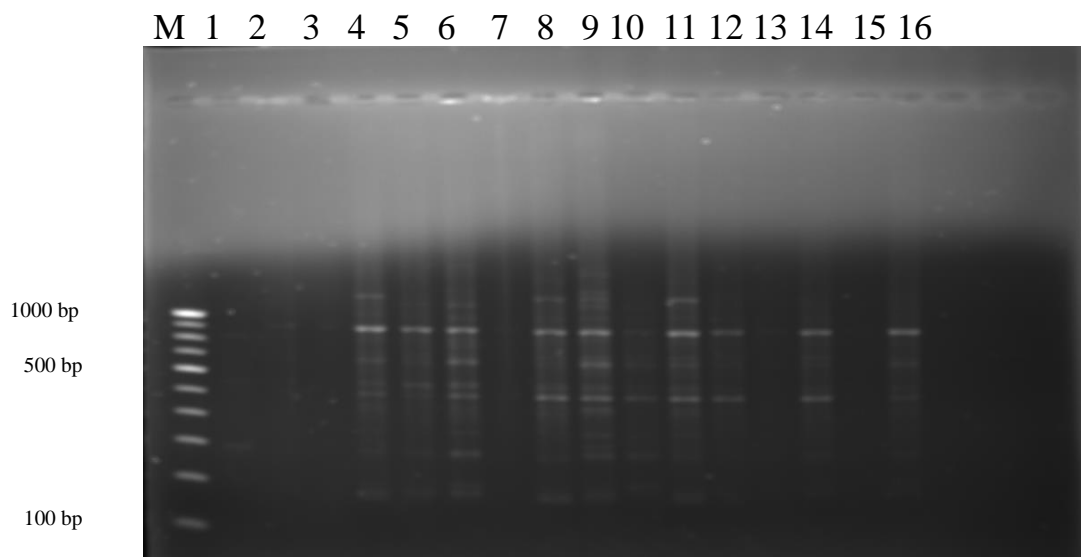


Figure 1 DNA Fingerprinting of somaclones with their parent varieties based on RAPD primer OPB-06 in 1.4% Agarose Gel. Lane M: Molecular weight marker (100bp DNA ladder); Lane 1: Variety Isd 37; Lane 2: Somaclone CC-37-1; Lane 3: Somaclone CC-37-4; Lane-4: Somaclone CC-37-5; Lane-5: Variety Isd 38; Lane-6: Somaclone CC-38-1; Lane-7: Somaclone CC-38-2; Lane-8: Somaclone CC-38-5; Lane-9: Variety Isd 39; Lane-10: Somaclone CC-39-3; Lane-11: Somaclone CC-39-5; Lane 12: Somaclone CC-39-6; Lane-13: Variety Isd 40; Lane-14: Somaclone CC-40-2; Lane-15: Somaclone CC-40-3 and Lane-16: Somaclone CC-40-6

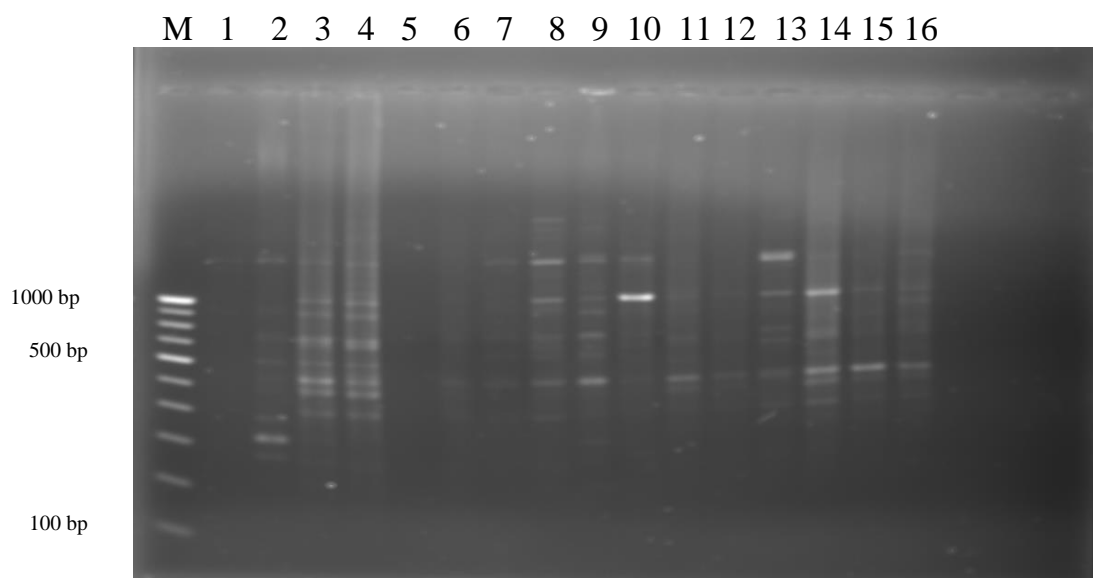


Figure 2 DNA Fingerprinting of somaclones with their parent varieties based on RAPD primer OPA-07 (GAA ACG GGTG) through 1.4% Agarose Gel. Lane M: Molecular weight marker (100bp DNA ladder); Lane 1: Variety Isd 37; Lane 2: Somaclone CC-37-1; Lane 3: Somaclone CC-37-4; Lane-4: Somaclone CC-37-5; Lane-5: Variety Isd 38; Lane-6: Somaclone CC-38-1; Lane-7: Somaclone CC-38-2; Lane-8: Somaclone CC-38-5; Lane-9: Variety Isd 39; Lane-10: Somaclone CC-39-3; Lane-11: Somaclone CC-39-5; Lane-12: Somaclone CC-39-6; Lane-13: Variety Isd 40; Lane-14: Somaclone CC-40-2; Lane-15: Somaclone CC-40-3 and Lane-16: Somaclone CC-40-6

dissimilarity percentage (91%) in Isd 37 vs CC-37-1. All somaclones and their parents showed high level of dissimilarity percentage ranged from 51 to 91 with average 74% and also similarity index ranged from 0.09 to 0.49 with average 0.26 among 12 genotype combinations. Information on the genetic

relationships among genotypes based on the molecular markers can help to decide which genotype is highly variable. Plant breeders can get the idea of breeding program and goal directed towards the selection of diverse parents to produce heterotic hybrid varieties. The reduced values of distance estimates

between the genotypes seemed to suggest a downward trend on the level of genetic diversity present in the genotypes evaluated. This can be further enhanced by the use of related or limited number of parents in the hybridization program. The presented tree diagram demonstrates clearly the ability of the 6 RAPD markers to detect a large amount of genetic variation in sixteen sugarcane genotypes. The results of the present investigation revealed that the six RAPD primers were able to identify and classify the sixteen sugarcane genotypes based on their genetic relationship. These similar type findings were recorded by Khan *et al.* (2010); Alvi *et al.* (2008); Ali *et al.* (2013) and Mahmud *et al.* (2015). Therefore, DNA fingerprinting and molecular relationship of all the varieties and their somaclones as well as entire sugarcane germplasm core collection should be done using RAPD markers in order to determine their genetic relationships for variety development. Nevertheless, it is also suggested that to get more precise results, the number of primers should be increased. Very limited reports on the use of DNA markers for the estimation of somaclonal variation are available. Hence this study can be used as a point reference for further studies.

CONCLUSION

It may be concluded that callus culture supplemented with 2,4-D can be used to create somaclonal variation along with molecular diversity which can be determined by using RAPD markers.

MATERIALS AND METHODS

The experiment was conducted at the Biotechnology Laboratory, Bangladesh Sugarcrop Research Institute (BSRI), Ishurdi, Pabna, Bangladesh during the period from 2010 to 2011 to obtain *in-vitro* plant regeneration potentiality of BSRI released varieties Isd 37, Isd 38, Isd 39 and Isd 40. The leaf sheath explants were collected from 8-10 months old field grown sugarcane from BSRI experimental field. At first MS medium supplemented with green coconut water (10%) containing 3 mg/l of 2, 4-D for callus induction. After five weeks, the calli were inoculated for shooting medium supplemented with concentration BAP (2 mg/l) + Kinetin (1 mg/l) and maintained by sub-culturing every two weeks and then regenerated shoots were inoculated for rooting by sub-culturing every two weeks on MS medium supplemented with 5 mg/l NAA. Rooted plantlets were acclimatized and transplanted to polybag and then field respectively. For molecular studies, young meristem cylinder from 12 somaclones and their donor parents were taken from R₀ regeneration for DNA isolation. DNA was extracted using modified and combined methods of Aljanabi *et al.* (1999) and mini-prep method adopted from Hossain *et al.* (2006) and Shahnawaz (2006). The DNA concentration was determined by Nano drop Spectrophotometer (2000/2000c, Thermo Scientific, USA) and was diluted to a concentration of 50 ng/μl. Samples were stored at -20°C for further use. Polymorphism was studied using Random Amplified Polymorphic DNA (RAPD) as illustrated by Mondal *et al.* (2009) which supported by Williams *et al.* (1990). Six RAPD primers (Operon Technologies, Inc., Alameda, California, USA) were used. List of polymorphic primers and their sequences are subsequently given. The reaction mixtures 10 μl was amplified for each DNA sample in a Thermal Cycler (Genius, Techne, Cambridge Limited). Agarose gel (1.4%, w/v) was used for RAPD electrophoresis. The Ethidium Bromide at 10

mg/ml was added in gel for detection of bands were viewed under ultraviolet trans-illuminator and documentation (FluorChem FC2, Cell Biosciences, USA) and also analysis system was used to make photographs. Besides, it was printed and saved on CD for lateral use.

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